Two Process Account of Aversive Classical Conditioning: Amygdala Modulation of Cerebellar-dependent Eyeblink Conditioning

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Abstract

In delay eyeblink conditioning (EBC) a neutral conditioned stimulus (CS; tone) is repeatedly paired with a mildly aversive unconditioned stimulus (US; eye-shock). After repeated CS-US pairings, subjects produce an eyeblink conditioned response (CR) to the CS independent of the US. Acquisition of the eyeblink CR relies on the cerebellum and brainstem. Early in training, the amygdala is proposed to rapidly form a CS-US association, resulting in the expression of multiple emotional (fear) CRs, including freezing. The central nucleus of the amygdala (CEA) is hypothesized to amplify CS responsiveness in the lateral pontine nuclei (LPN), which relays sensory information to the cerebellum, promoting more robust synaptic plasticity and learning. The CEA was bilaterally lesioned in order to assess the consequences on emotional and motor learning. Lesions were predicted to reduce fear responding (freezing), lower CS reactivity in the pons, and impair delay EBC acquisition. One week after surgery, rats experienced 1 or 4 sessions of delay EBC. Freezing behavior, eyeblink CR acquisition, and the amplitude of the unconditioned eyeblink response (UR) were measured across each session. At the end of training, rats were sacrificed and the extent of the CEA lesion was quantified via NeuN immunohistochemistry. Pontine nuclei activation was quantified via detection of c-Fos, an immediate early gene preferentially expressed in spiking neurons. Behavioral data demonstrates reduced levels of freezing, slower CR acquisition, and lower UR amplitudes in CEA lesioned subjects. Results support two process accounts of aversive associative learning, which posit that amygdala-dependent emotional CRs influence or modulate acquisition of the cerebellar-dependent motor (eyeblink) CR.

Introduction

Various forms of Pavlovian conditioning have long been established to rely on different neural substrates and circuitry (Kim and Baxter, 2001). For instance, classical eyeblink conditioning (EBC) is critically reliant on the brainstem and cerebellum (Steinmetz and Lindquist, 2009), whereas fear conditioning depends on the amygdala (LeDoux, 2000). Two process accounts of aversive conditioning postulate that non-specific emotional responses proceed and modulate the generation of specific motor responses (Konorski, 1967; Rescorla and Solomon, 1967). Specifically, during EBC the rapidly acquired emotional fear conditioned response (CR) is proposed to influence or modulate acquisition of the more slowly acquired eyeblink motor CR (Lee and Kim, 2004; Mintz and Wang-Ninio, 2001). To further delineate the circuitry by which this proposed two process mechanism occurs, we utilized the EBC paradigm. Eyeblink conditioning acts as an exemplar of this model due to its anatomically and behaviorally dissociable responses, control of experimental stimuli, and the ability to accurately measure behavioral responses. Additionally, decades of research utilizing the EBC paradigm have made it arguably the best understood form of Pavlovian conditioning in terms of behavior and neural circuitry (Christian and Thompson, 2003).

During delay EBC, repeated pairings of a neutral conditioned stimulus (CS; tone) with a mildly aversive unconditioned stimulus (US; periorbital shock) leads to a learned association. Over training, the eyeblink CR emerges in response to CS presentation, with the conditioned blink being generated just before US onset (Lindquist et al. 2009). Dependent on both sensory and motor pathways, EBC acquisition and expression is critically reliant on the brainstem and cerebellum (Steinmetz and Lindquist, 2009). The cerebellum is an essential structure in delay EBC and alone is capable of encoding and storing the eyeblink CR in decerebrated rabbits

(Mauk and Thompson, 1987). The US (eye-shock) pathway to the cerebellum involves climbing fiber projections from the inferior olive, which synapse onto Purkinje cells in cerebellar cortex. The CS (tone) input to the cerebellum relies on the lateral pontine nucleus (LPN). Selective lesions of the LPN abolish the CR when the CS is an auditory cue (Steinmetz et al., 1987), identifying the LPN as a mediator of the auditory CS pathway to the cerebellum. Within the cerebellum, neuronal recordings of the interpositus nucleus (IP) during EBC display learning induced increases in neuronal unit activity correlated with production of the eyeblink CR (Christian and Thompson, 2003), implicating the IP as the site of CS-US convergence.

Although the brainstem-cerebellar neural circuit is necessary and sufficient for EBC acquisition, this is not exclusive, and other brain regions can influence and modulate the acquisition rate of the eyeblink CR (Lee and Kim, 2004; Mintz and Wang-Ninio, 2001). For example, the amygdala is located deep within the medial temporal lobe of the brain and is part of the emotional limbic system. It encodes and stores emotional memories and modulates the memory stored in other structures (McGaugh et al., 2002), including the cerebellum in EBC. This limbic structure is anatomically differentiated into multiple regions including basolateral amygdala (BLA) and central nucleus of the amygdala (CEA) that have distinct connectional and functional characteristics (Lindquist and Brown, 2004). Essential for fear conditioning, CEA is proposed to generate a variety of fear CRs via output projections to the brainstem, hypothalamus, and medulla (Hopkins and Holstege, 1978; LeDoux et al., 1988). As a result of the aversive nature of the eye-shock US in EBC, there are multiple fear responses including freezing, 22 kHz vocalizations, and reflex facilitation (Lee and Kim, 2004; Lindquist and Brown, 2004). The acquisition of delay EBC is slowed in amygdala lesioned rabbits and rats (Blankenship et al., 2005; Lee and Kim, 2004; Mintz and Wang-Ninio, 2001; Weisz et al., 1992).

It is known that the amygdala facilitates cerebellar motor conditioning (Lennartz and Weinberger, 1992; Thompson et al., 1987), although the mechanism by which this is achieved has yet to be defined. A possible mechanism, the CEA, is known to potentiate or enhance certain reflexes (Choi et al., 2001) and to amplify reflexive eyeblink unconditioned responses (URs) when stimulated electrically (Whalen and Kapp, 1991). Inducing the many fear CRs early in training, the CEA may also amplify CS reactivity through monosynaptic or polysynaptic projections to the LPN (Holstege et al., 1986a; Holstege et al., 1986b). This connection may enhance the saliency of the CS signal, accelerating CS-US associative plasticity within the IP (Taub and Mintz, 2010).

The present study investigated the two process account of aversive conditioning in adult male rats to elucidate the mechanism by which the emotional CR facilitates the acquisition of the motor CR. We hypothesized that the CEA modulates the acquisition of the eyeblink CR through enhanced synaptic plasticity within the IP due to CEA facilitation of the PN throughput of the auditory signal (see Figure 1). To investigate, the CEA was bilaterally lesioned in rats submitted to delay EBC. To measure the effect of the CEA lesions throughout training, we examined three dependent variables. Freezing was measured along with the acquisition of the eyeblink CR in all subjects. The amplitude of the eyeblink UR was also measured, which can be modified as a function of learning during delay EBC. In addition, all rats were submitted to one or four EBC training sessions in order to account for the variability in the acquisition rate of emotional CRs and motor CRs.

In accordance with previous studies and the two process account of aversive conditioning, the emergence of fear CRs occurred early in training and preceded the eyeblink CR. Rats were sacrificed 1 h following one or four EBC training sessions and

immunohistochemistry (IHC) was performed in the LPN via c-Fos, an immediate early gene expressed as a consequence of neuronal activity. c-Fos expression was quantified in order to assess CS sensory reactivity between rats with an intact or chemically lesioned CEA. Results indicate less c-Fos activation in the lesioned rats. These results support the two process account of aversive conditioning, and offer a suggestion as to how the amygdala might modulate or regulate eyeblink CR acquisition.

Methods

Subjects. Subjects were 30 male Long Evans rats purchased through an official dealer, Harlan Laboratory. All rats were maintained with *ad libitum* feeding under a 12/12 h light/dark cycle. All procedures used in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Surgery. One week after arrival, subjects underwent survival surgery. Rats were anesthetized with respiratory administration of 1-3% isoflurane throughout surgical procedures. After deep anesthesia was achieved, monitored by toe pinch, subjects were positioned in a stereotaxic frame. Using the stereotaxic instrument, bilateral lesions aimed at the CEA were achieved using the following coordinates, relative to bregma (in mm); AP: -1.5, ML: ±4.3, DV: -8.2; and AP: -2.4, ML: ±4.3, DV: -8.5. Coordinates were based on the atlas of Paxinos and Watson (1998). The two drop sites ensured the entire AP extent of the CEA was damaged. Bore holes were drilled through the skull with a dental drill to allow the passage of a Hamilton syringe into the CEA. Chemical lesions were bilaterally administered to CEA lesion subjects (n=15) using 0.1µg/µl ibotenic acid infused over 1 minute. Sham lesion controls (n=15) received bilateral administration of PBS vehicle. In order to deliver a periorbital electrical eye-shock, all subjects

received a subdermal implantation of a bipolar electrical stimulator near the dorso-caudal region of the left eye. Electromyographic (EMG) activity was recorded in the orbicularis oculi muscle by passing two thin (0.003 in. bare) differential EMG wires subdermally through the upper eyelid. EMG wires were contained within gold pins mounted to a plastic pedestal (headstage) affixed to the skull with dental cement. The incision site was sutured after implantation and the wound was treated with an antibiotic. Subjects were given one week to recover before training began.

Apparatus. Operant conditioning chambers consisted of two stainless steel walls, two Plexiglas walls, and a grid floor composed of 0.5 cm stainless steel bars placed approximately 1.5 cm apart. Prior to training sessions, the operant conditioning chambers were sprayed with Windex®. Electrode leads, attached to each subject's head, swivel freely on a multi-channel commutator connected to a counterbalanced pivoting arm, allowing subjects to move freely about in the conditioning chamber during training sessions.

Behavioral Procedures. Subjects were given approximately one week to recover from surgery before behavior training began. Initially, each rat was habituated to the conditioning chamber for 60 minutes while its headstage was connected to the commutator. Half of all subjects received one training session and the other half received four training sessions. Delay EBC was administered using a 85 dB, 450 msec tone CS and a co-terminating 100 msec, 2.0 mA eye-shock. The 350 msec period separating the CS and US onset is called the interstimulus interval (ISI). Each training session consisted of 10 blocks of 12 trials: 10 CS-US paired, 1 CS-alone, and 1 US-alone. The intertrial interval (ITI) was 25 ± 5 sec.

Histology. Subjects were sacrificed 1 h after the last training session using Euthasol and subsequently perfused with 4% paraformaldehyde. Two days later the brains were transferred and fixed in 4% paraformaldehyde / 30% sucrose solution for one week. Tissue was sectioned at 40 μm across the amygdala and pons with a vibrating microtome. Tissue was stored in cryoprotectant until the start of immunohistochemistry (IHC).

To determine the placement of lesions, NeuN (a stain specific to neurons) was used to quantify the extent of excitotoxic lesions of the CEA. The NeuN antibody was obtained from EMD Millipore Inc., Darmstadt, Germany. Tissue samples were quenched in 0.3% H2O2 and then blocked in 5% normal goat serum for one hour. Sections were incubated overnight at 4°C with 1/200 dilution of NeuN antibody. The next day, sections were incubated in 1/200 dilution of biotinylated anti-mouse antibody for one hour at room temperature. ABC and DAB kits were used before mounting the sections onto slides. Images of the CEA lesion for each subject were acquired using the Nikon 90i scope at 10X magnification. Lesion reconstructions were done based on three plates (-1.80, -2.56, and -3.14) from Paxinos and Watson which cover the entire extent of the amygdala. Under 2X magnification, bilateral lesions were drawn onto each plate. Next, NIS-Elements software was used to determine the region of interest (ROI) of the CEA in each plate, followed by an outline of each lesion. This allowed us to compute the percentage of CEA damage for each bilateral section across each lesion subject.

To determine neuronal activity within the lateral pons, c-Fos IHC staining procedures were used. Sections were quenched in 0.3% H2O2 and then blocked in 3% normal goat serum for one hour. Sections were then incubated for 48 h at 4°C with a rabbit polyclonal antibody against the Fos protein (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1/2000 in 3% normal goat serum. Sections were then incubated at room temperature for two

hours with a biotinylated goat anti-rabbit immunoglobulin diluted 1/200 and for one hour with a standard ABC kit. Before mounting sections onto slides, a DAB kit was used. Images of the LPN for each subject were acquired using the Nikon 90i scope at 10X magnification. Nikon-Elements software was used to draw ROIs around the LPN in 1-3 sections per animals. Within each ROI, stained cells were counted in a blind manner across all sections. In each hemispheric section, LPN cell counts were divided by the ROI area to generate the number of cells per mm^2.

Data analysis. Throughout habituation and EBC, motion sensitive cameras recorded freezing behavior. The video signal recorded with black-and-white cameras was inputted to Freezescan, a program that can detect cessation of movement. Eyeblink analysis (CR percentage and UR amplitude) was computed on the basis of rectified raw EMG signals on each trial and analyzed using proprietary spike2 software. Collected data was analyzed using multiple one-way, two-way, and repeated measures ANOVAs.

Results

Lesion Reconstructions. Across all lesion subjects, approximately 55.8% of the CEA was damaged in both hemispheres. The damage did vary across the AP axis, with 35.2% damage at plate -1.80, 51.1% damage at plate -2.56, and 81.0% damage at plate -3.14 (Figure 2A). A total of four CEA lesion subjects were dropped due to incomplete lesions, defined as less than 50% across all three plates. Figure 2B illustrates photo-micrographs of two representative lesions in the left and right hemisphere.

Behavioral analysis Figure 3 displays the percentage of freezing behavior recorded in CEA lesion and sham rats that received one or four training sessions. One-way (Surgery) ANOVA revealed that in rats that received one training session the CEA lesion subjects produced

significantly less freezing behavior, $F(1,11) = 8.90$, $p < 0.05$, than sham subjects. In rats trained with four sessions, repeated measures ANOVA (with one between-subject factor, surgery, and one within-subject factor, session) indicated the lesion subjects were marginally impaired in freezing behavior ($p = 0.06$) compared to sham subjects. No significant effect was seen for session or the surgery x session interaction. Taken together, these results indicate CEA lesions diminished expression of the emotional CR.

Figure 4 displays the percentage of eyeblink CRs recorded in lesion and sham groups that received either one or four training sessions. As expected, the one-way ANOVA revealed no significant surgical effect in subjects that received one training session ($p = 0.90$). Across the four training sessions, the CEA lesion rats showed a trend toward fewer eyeblink CRs relative to sham subjects, though the effect only approached statistical significance as revealed by the repeated measures ANOVA ($p = 0.11$). No significant effect was seen for session or the surgery x session interaction.

Figure 5 depicts the amplitude of the eyeblink UR across one or four training sessions in sham and lesion subjects. While the one-way ANOVA revealed no surgical effect between subjects that received one training session ($p = 0.78$), repeated measures ANOVA showed a trend towards lower amplitude in lesion subjects across the four training sessions ($p = 0.15$). No significant effect was seen for session or the surgery x session interaction.

Immunohistochemical Analysis. Neuronal spiking within the LPN was quantified via the expression of c-Fos+ cells. The expression of c-Fos was examined with a two-way ANOVA, consisting of two between-subject variables, surgery and session. Results revealed a significant main effect for surgery, $F(1,100) = 8.26$, $p < 0.001$. The session effect nor the surgery x session

interaction reached significance (Figure 6A). Collapsing across training session, the sham subjects had significantly more c-Fos+ cells than the CEA lesion rats, $F(1,102) = 7.39$, $p < 0.01$, as revealed by a one-way ANOVA (Figure 6B).

Discussion

The current results are in accord with the two process account of aversive classical conditioning and identify a possible mechanism by which the amygdala modulates cerebellar function during delay EBC. Results indicate that CEA lesions reduced freezing behavior (Figure 3), diminished eyeblink CR acquisition (Figure 4), and restricted learning-dependent increases in the eyeblink UR (Figure 5). Altogether, the results support the notion that the CEA modulates motor (eyeblink) learning. The reduction in amygdala-dependent fear expression (freezing) and decreased modulation of the UR amplitude indicate that the lesions successfully impaired CEA output. Accordingly, delay EBC was also impaired as a consequence of the CEA lesion. As illustrated in Figure 1, these results are consistent with our hypothesis that one role of the CEA in EBC is to amplify throughput of the CS signal on its way to the cerebellum.

Both eyeblink and fear Pavlovian conditioning have been extensively studied, but mostly independently. As discussed in the Introduction, delay EBC is cerebellar-dependent and, across training, the CS becomes temporally predictive of US onset and subjects produce eyeblink CRs with maximal eyelid extension occurring just before US onset. CS and US input to the cerebellum is achieved via granule cells and inferior olive cells, respectively. The CS and US signals converge onto neurons in the interpositus (IP) nucleus and onto Purkinje cells in the cerebellar cortex (Steinmetz and Lindquist, 2009). The cerebellar cortex is proposed to modulate the conditioned blink's topography—regulating CR amplitude and timing. The critical CS-US

plasticity is thought to occur in the IP where populations of cells have been recorded that fire just before onset of the eyeblink, modeling the behavioral responses amplitude and timing (Freeman and Nicholson, 2000; McCormick et al., 1983; Rogers et al., 2001). Learning-dependent increase in IP spiking to the (tone) CS is proposed to activate downstream motor nuclei which drive production of the conditioned blink (Thompson, 2005).

The amygdala also has a well-established role in emotional learning. Located within the medial temporal lobe, it is critical for the development and expression of conditioned fear (Brown et al., 2003; Steinmetz and Lindquist, 2009). The amygdala can be divided into functionally and anatomically distinct nuclei (Swanson and Petrovich, 1998). During classical eyeblink conditioning, CS and US sensory information enters the amygdala through the BLA, which encodes and associates the CS and US within the first training session, resulting in the rapid expression of various fear CRs. The BLA projects to the CEA, which governs fear expression. The CEA, in turn, projects to a variety of brainstem and hypothalamic regions responsible for conditioned fear, including freezing behavior (LeDoux, 2000).

Our lab is interested in characterizing the relationship between these two anatomically distinct learning and memory systems, fear and eyeblink conditioning. In fact, ideas concerning this relationship have a long history, including two process theories (Konorski, 1967; Lee and Kim, 2004; Mintz and Wang-Ninio, 2001; Rescorla and Solomon, 1967; Thompson et al., 1987; Wagner and Brandon, 1989). In two process accounts of aversive classical conditioning, rapidly acquired non-specific emotional responses, including freezing, influence or modulate the learning rate of specific motor (eyeblink) responses (Mintz and Wang-Ninio, 2001; Thompson et al., 1987). The two process account is based on the idea that the aversive US can be dually represented as both “emotional” and “sensory” (Lindquist et al., 2010; Wagner and Brandon,

1989). Thus, these dually represented US attributes, emotion and sensation, can be independently associated with the CS. In the current experiment, the eye-shock (US) is dually represented as "emotional" and is amygdala-dependent while concurrently the US is "sensory" and cerebellar-dependent. Previous studies that have used amygdala lesions or inactivation have revealed diminished eyeblink CR acquisition (Blankenship et al., 2005; Lee and Kim, 2004; Weisz et al., 1992). In no case did the loss of the first process prevent learning by the second process, but there was no facilitated learning (Chachich and Powell, 1998). This result indicates that the amygdala contributes to but is not necessary for the development and generation of the eyeblink CR. In support, Rorick-Kehn and Steinmetz (2005) suggest the CEA associates the CS and US and increases arousal or directs attention to the CS via output connections to the brainstem, hypothalamus, and other brain regions. Based on these data and ideas, we hypothesized that the CEA—as part of its many output projections—also facilitates CS responsiveness in the PN, potentially facilitating CS-US associative plasticity within the IP.

To further elucidate the neural circuit involved in the two process account of conditioning, we investigated the consequence of bilateral CEA lesions on the acquisition of the (eyeblink) CR in delay EBC. As expected, the freezing behavior in sham rats peaked during the first training session and decreased thereafter, whereas the eyeblink CR was lowest in the first training session and increased across subsequent sessions (Figure 3). These general CR acquisition trends are in accordance with the two process account of aversive conditioning (Konorski, 1967; Rescorla and Solomon, 1967), and exemplify the amygdala's importance in the emergence of non-specific CRs (e.g., bradycardia, 22 kHz ultrasonic vocalizations, and freezing behavior), supporting the amygdala's role in rapid emotional learning.

Rats that received CEA lesions demonstrated less freezing and slower eyeblink CR acquisition (Figures 3 & 4). Sham and lesion groups trained for one session showed no significant difference in eyeblink CR, but a robust difference in freezing. This effect was expected because freezing is amygdala-dependent. Across the four training sessions, the lesion rats learned more slowly, again supporting the idea that the CEA contributes to enhanced eyeblink conditioning.

As illustrated in Figure 5, the UR amplitude is lower in CEA lesion rats compared to controls. The amygdala is known to facilitate the eyeblink UR as a function of eyeblink CR acquisition. CS-mediated UR facilitation occurs within 5-10 trials and is thought to be the product of CEA modulation of the UR pathway (Weisz and McInerney, 1990). UR facilitation can also occur independent of the CS, as assessed by US-alone trials. Conditioning-specific reflex modification (CRM), as the latter is known (Burhans et al., 2008), reflects UR facilitation independent of the CS. An associative form of learning, it develops concurrent with the eyeblink CR and is characterized by increases in the amplitude and/or area of the nictitating membrane UR in rabbits (reviewed in Schreurs, 2003). CEA inactivation during US-alone testing abolishes CR-mediated facilitation. Facilitation remains intact, however, if the CEA is inactivated during delay EBC, suggesting the CEA is involved in CRM expression but not its acquisition (Burhans and Schreurs, 2008). The CRM might depend on learning-related excitatory unit activity in the IP, with its reversible inactivation during US-alone trials reducing UR amplitudes (Wikgren et al., 2002), though exactly how the IP might regulate CEA output remains unclear. Whalen and Kapp (1991) suggest that CEA projections to the lateral tegmental field (LTF) of the brainstem contribute to increased arousal and UR amplitudes. The fact that no learning-dependent increases in UR amplitude are seen across the four EBC sessions in the CEA lesioned rats again

supports our hypothesis that the amygdala modulates the EBC neural circuit as a consequence of IP-dependent learning.

In addition to behavioral testing, which was used to demonstrate improper fear and eyeblink conditioning as an effect of CEA lesions, we also performed IHC to quantify neuronal activity within the LPN. IHC staining for c-Fos revealed decreased neuronal activation within the LPN of CEA lesion subjects compared to sham controls. CS information reaches the IP through the PN. It is proposed that in lesion subjects the PN lacks CEA amplification of the auditory throughput and as consequence less neuronal activation is observed. This would, presumably, then lead to less IP plasticity due to the diminished CS signal seen by IP. Nevertheless, the behavioral and IHC data are congruent—indicating less LPN reactivity to the tone CS in lesion subjects, accompanied by reduced freezing, motor CR learning, and associative changes in UR amplitude.

In conclusion, the current experiment adds further support to the two process account of aversive classical conditioning. As part of this thesis' future publication, c-Fos+ cells will be quantified in the BLA and CEA of sham rats (Figure 7). The IHC results are expected to provide insight into the neuronal responsiveness of each amygdala nucleus. While our focus has been on CEA we cannot rule out a role for the BLA in modulating the acquisition and expression of the eyeblink CR. Indeed, previous work has demonstrated retarded delay EBC following BLA lesions or inactivation (Blankenship et al., 2005; Lee and Kim, 2004). Chemical CEA lesions were intentionally used in the current study in order to spare fibers of passage. Thus, it is possible BLA projections that run through the CEA could also play a role. Quantifying neuronal activity in both the BLA and CEA after one or four EBC sessions should help us clarify the role of each nucleus across conditioning.

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Figure Captions

Figure 1. Circuit diagram illustrating amygdala-dependent emotional learning and cerebellar-dependent motor learning. CS and US sensory information is relayed and associated in both the amygdala and cerebellum, and each produces its own CRs. The amygdala is proposed to process the "emotional" attributes of the US whereas the cerebellum is thought to process the "sensory" attributes of the US. The central nucleus of the amygdala (CEA) is hypothesized to amplify CS reactivity in the pons (red lines) via a monosynaptic or polysynaptic projection via the periacqueductal gray (PAG). The CEA may also modulate the eyeblink UR via polysynaptic projections through the PAG to brainstem motor nuclei, including the lateral tegmental field. As EBC progresses, the IP is proposed to provide its own positive feedback onto the pons (blue line) supplementing or replacing CEA activation.

Figure 2. Lesion reconstruction of CEA ibotenic lesions. (**A**) Reconstructions based on three atlas plates (-1.80, -2.56, and -3.14) from Paxions and Watson (1998). Across all lesioned subjects, the largest lesions are shown in gray and the smallest lesions are shown in black. (**B**) Representative photographs of CEA ibotenic acid lesions in the left and right hemispheres.

Figure 3. Freezing behavior (mean ± SE) in lesion and sham subjects submitted to one or four EBC sessions. Freezing was significantly reduced in lesion subjects trained with one session (far left). In subjects trained in four sessions, the difference in freezing between sham and lesion subjects approached but did not reach significance. Nevertheless, the lesion subjects do freeze less overall, suggesting the CEA is required for normal levels of fear responding.

Figure 4. Percentage of eyeblink CRs (mean ± SE) in lesion and sham subjects submitted to one or four EBC sessions. No differences were observed in sham and lesion rats trained with one

session (far left). Across the four training sessions, the lesion rats produced fewer CRs than sham rats. The results replicate previous findings that CEA perturbation disrupts the eyeblink CR acquisition rate.

Figure 5. Peak amplitude of the unconditioned eyeblink response (mean ± SE) in lesion and sham subjects submitted to one or four EBC sessions. No significant differences were observed with either training protocol. However, in rats trained with four sessions there is an increase in the reflexive blink's amplitude in sham rats. The lesion rats, on the other hand, demonstrate a flat amplitude across the four training sessions, suggesting the CEA is required for conditioning-specific reflex modification (CRM).

Figure 6. c-Fos+ cells (mean ± SE) in the LPN of lesion and sham subjects submitted to one or four EBC sessions. (**A**) c-Fos+ cells were significantly elevated in the sham rats relative to lesion rats after one, but not four, EBC sessions. (**B**) Collapsed across number of training sessions, the sham rats again displayed significantly more c-Fos+ cells than the CEA lesion rats. (**C**) Representative photographs of c-Fos+ cells in the LPN of a sham (top) and lesion (bottom) rat.

Figure 7. Representative photograph demonstrating c-Fos+ staining in the basolateral complex (BLA) and central nucleus (CEA) of the amygdala in one sham rat.

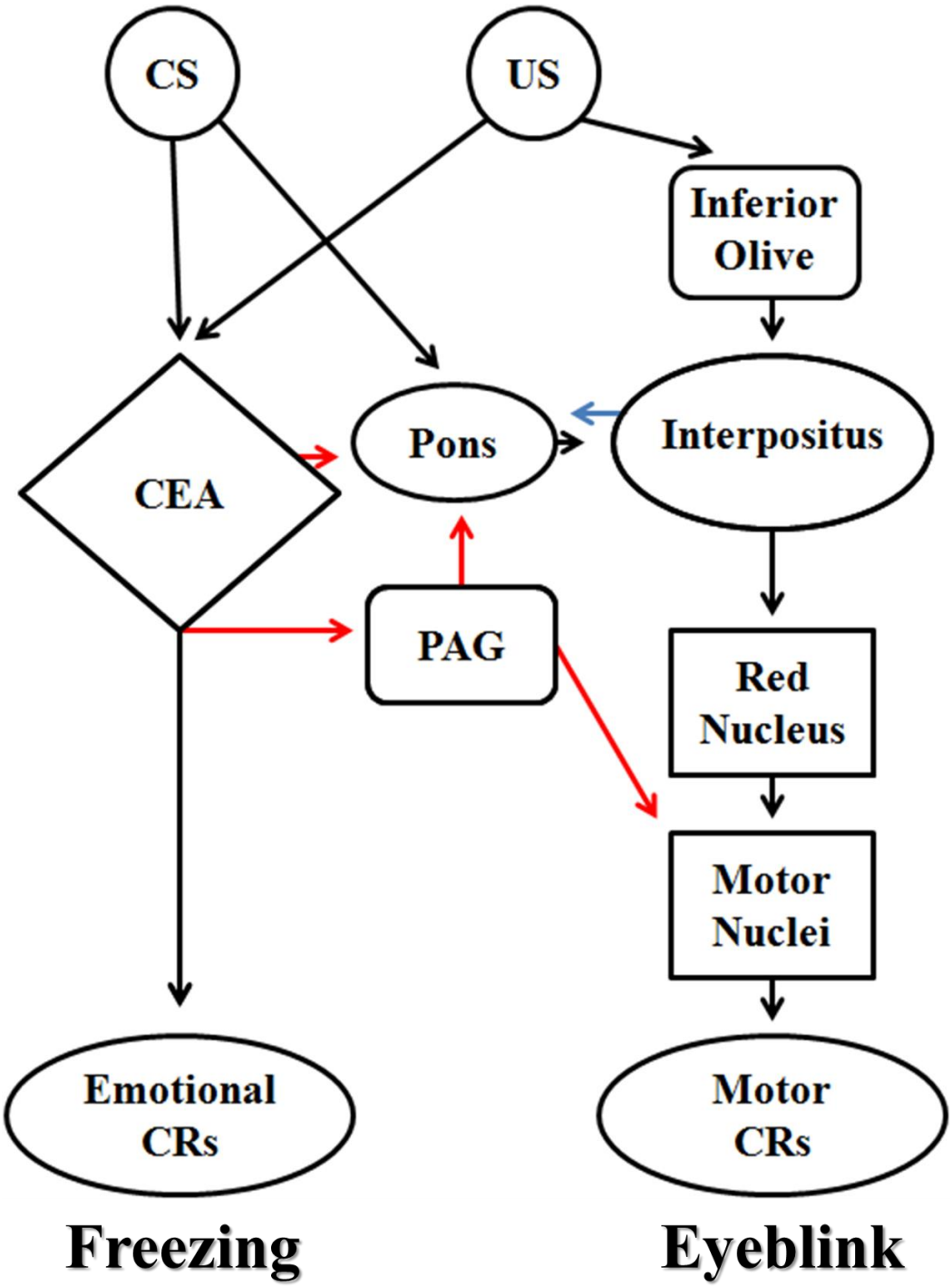

CS
US
Inferior Olive
CEA
Pons
Interpositus
PAG
Red Nucleus
Motor Nuclei
Emotional CRs
Motor CRs
Freezing
Eyeblink

Figure 1

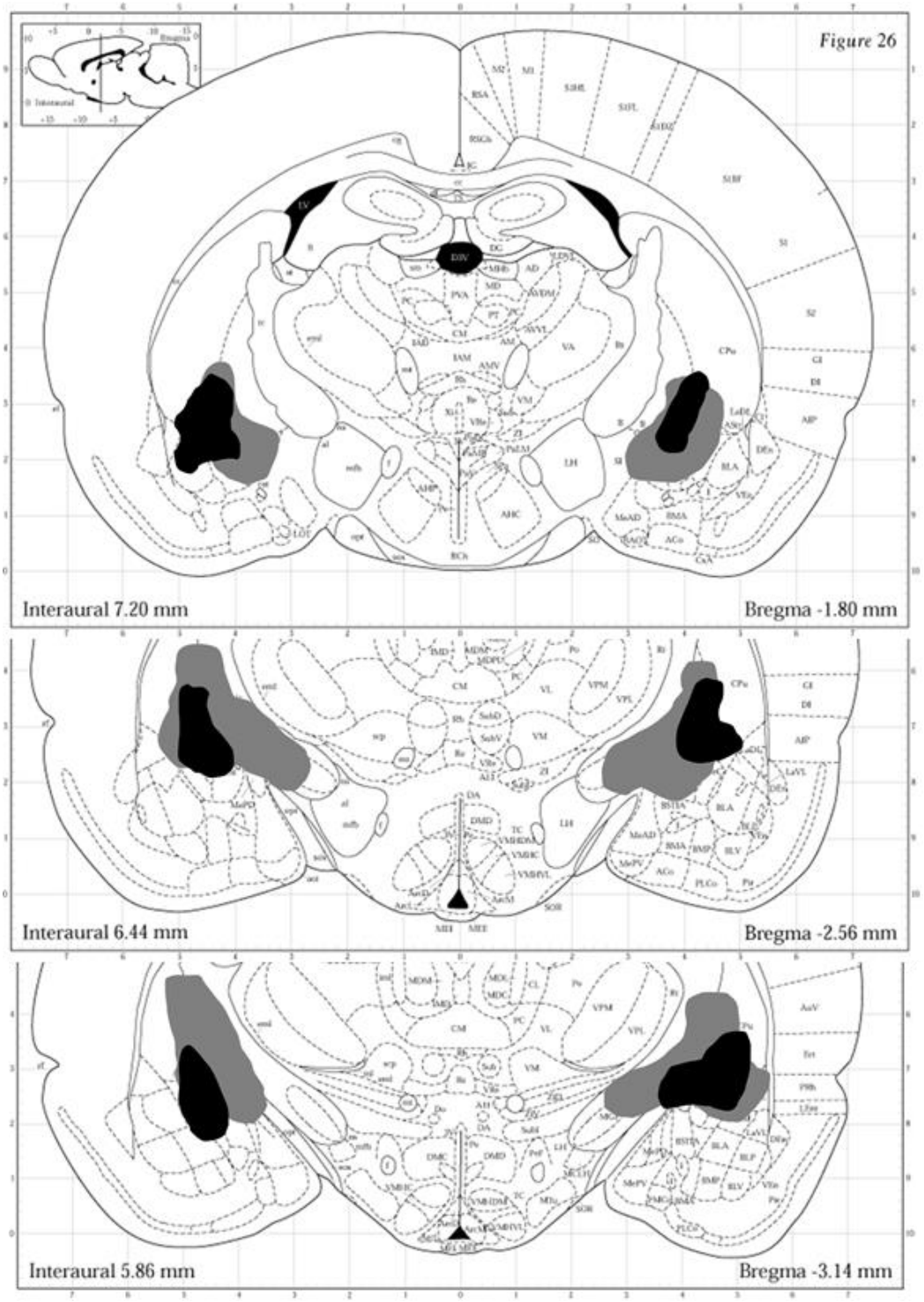


Figure 26
Interaural 7.20 mm
Bregma -1.80 mm
Interaural 6.44 mm
Bregma -2.56 mm
Interaural 5.86 mm
Bregma -3.14 mm

Figure 2

B

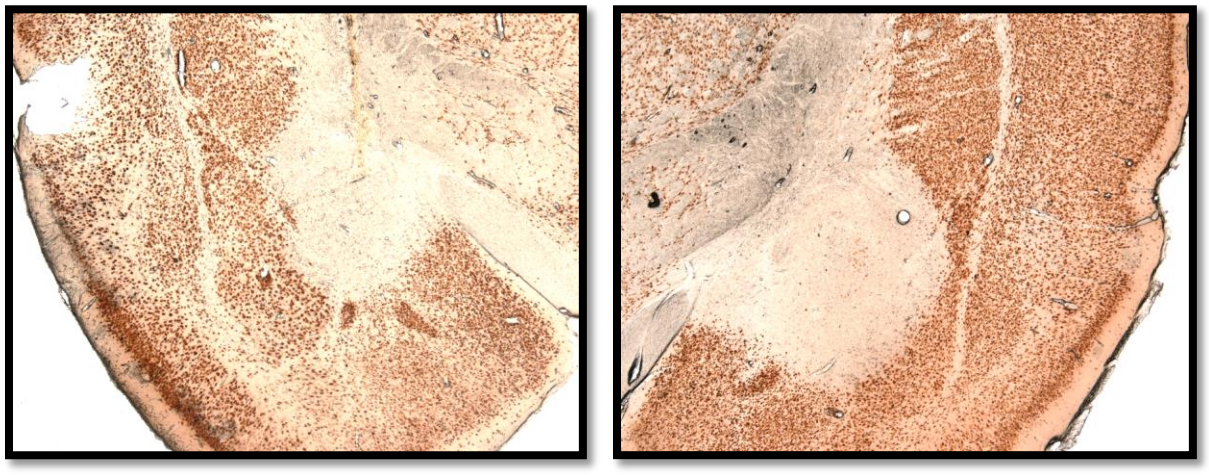


Figure 2

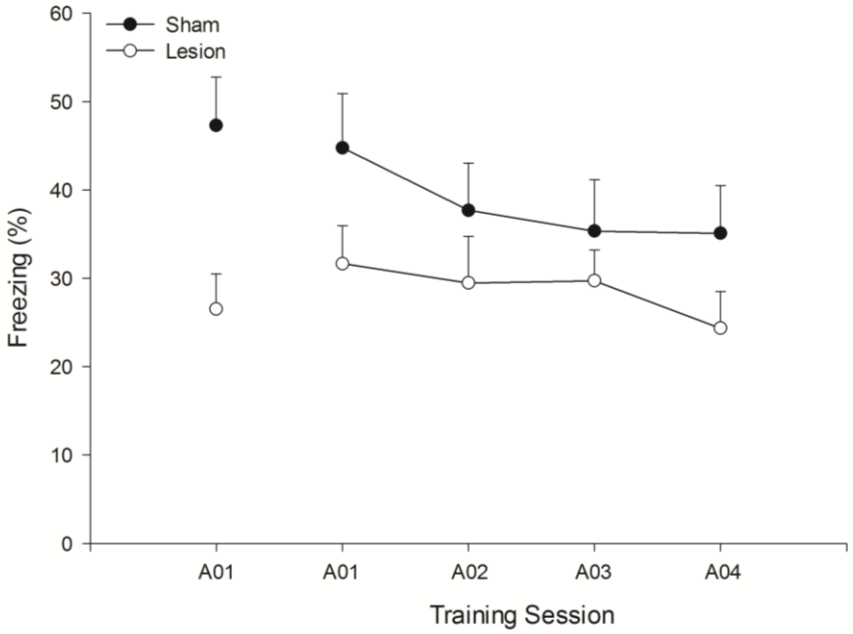

Sham
Lesion
Freezing (%)
60
50
40
30
20
10
0
A01
A01
A02
A03
A04
Training Session

Figure 3

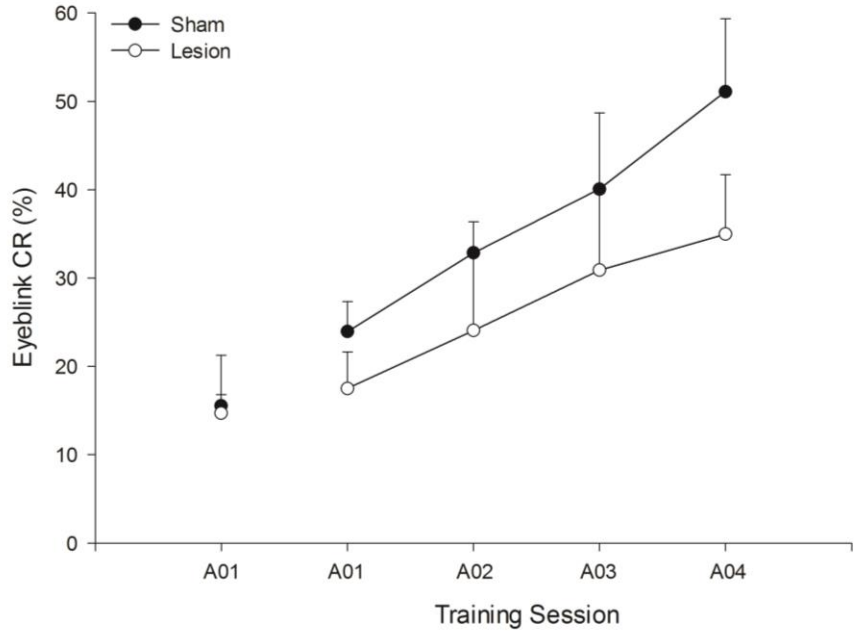

Sham
Lesion
Eyeblink CR (%)
60
50
40
30
20
10
0
A01
A01
A02
A03
A04
Training Session

Figure 4

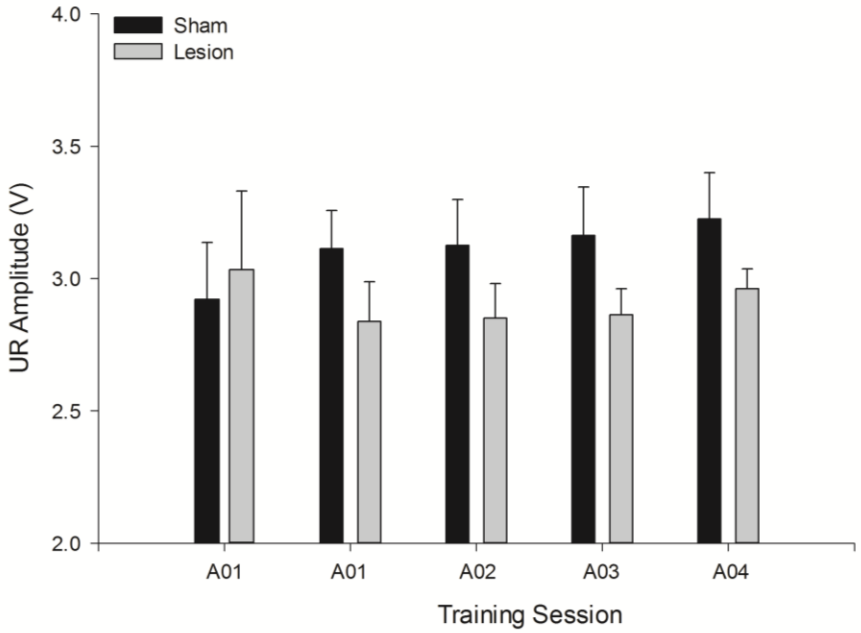
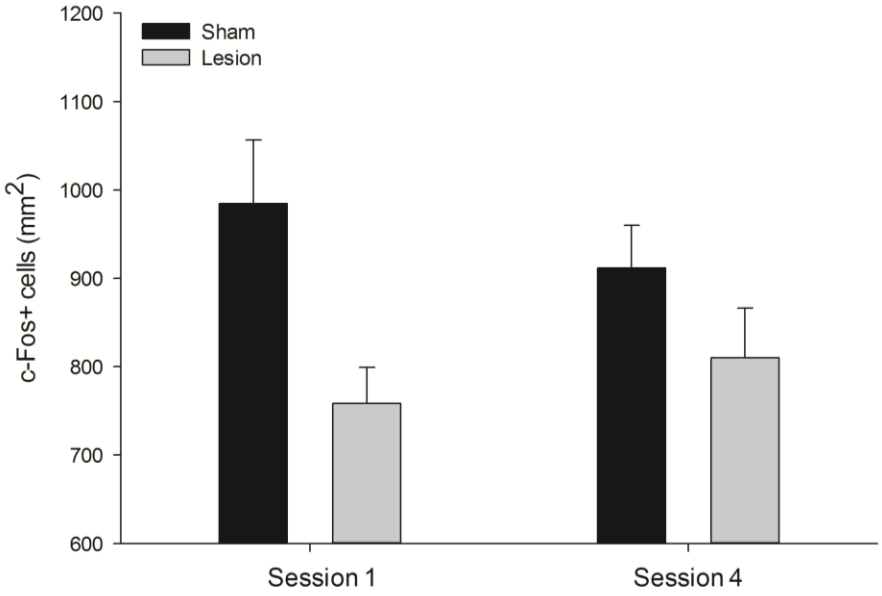

Sham
Lesion
4.0
3.5
3.0
2.5
2.0
UR Amplitude (V)
A01
A01
A02
A03
A04
Training Session

Figure 5

A


Sham
Lesion
1200
1100
1000
900
800
700
600
c-Fos+ cells (mm2)
Session 1
Session 4

B

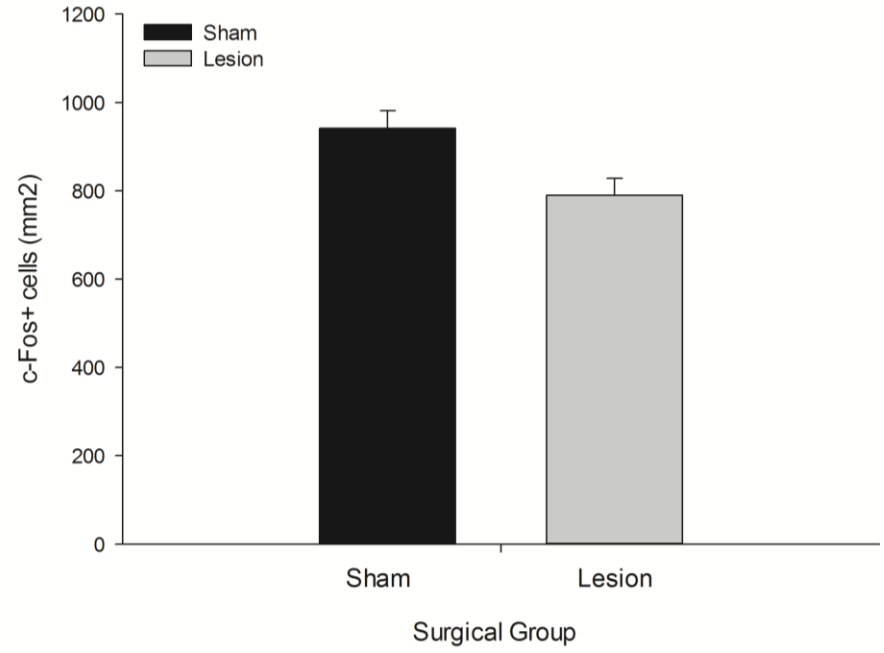

Sham
Lesion
1200
1000
800
600
400
200
0
c-Fos+ cells (mm2)
Sham
Lesion
Surgical Group

Figure 6

C

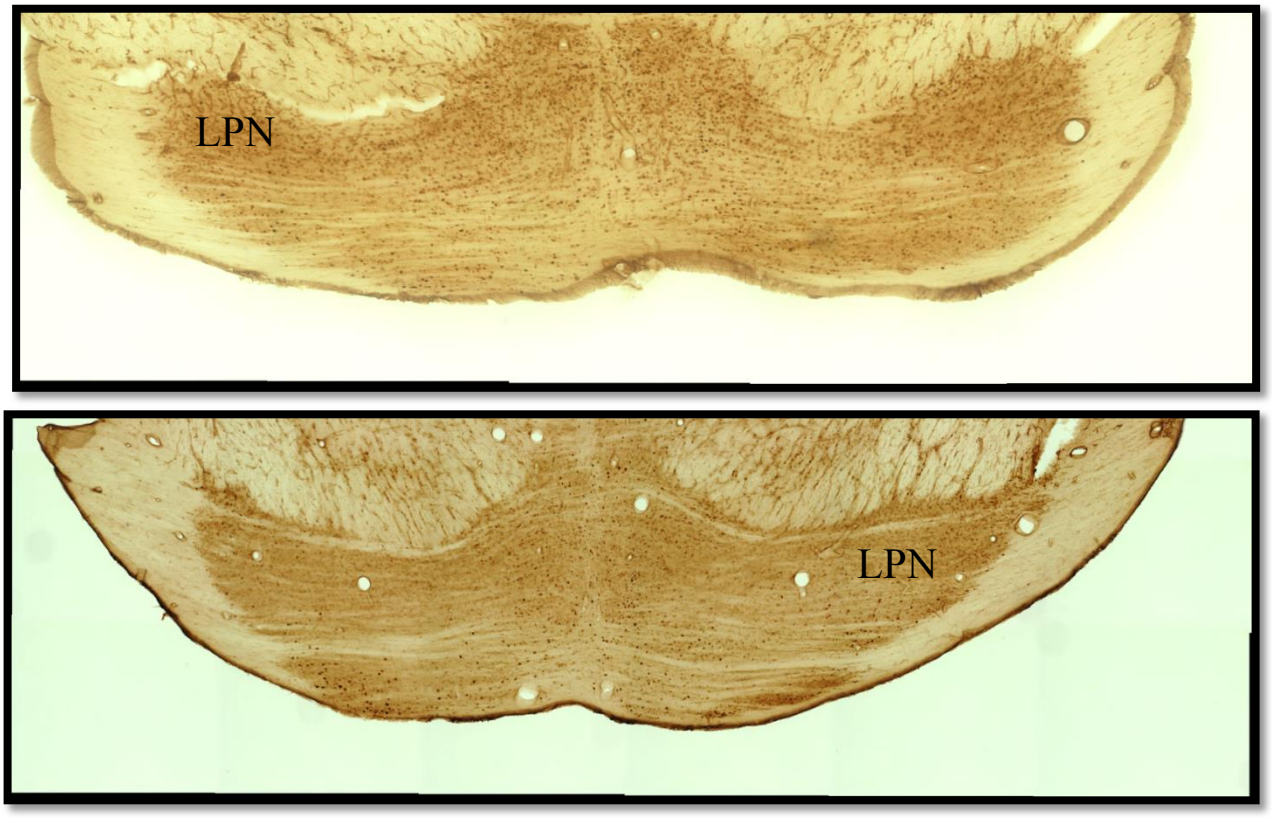


Figure 6

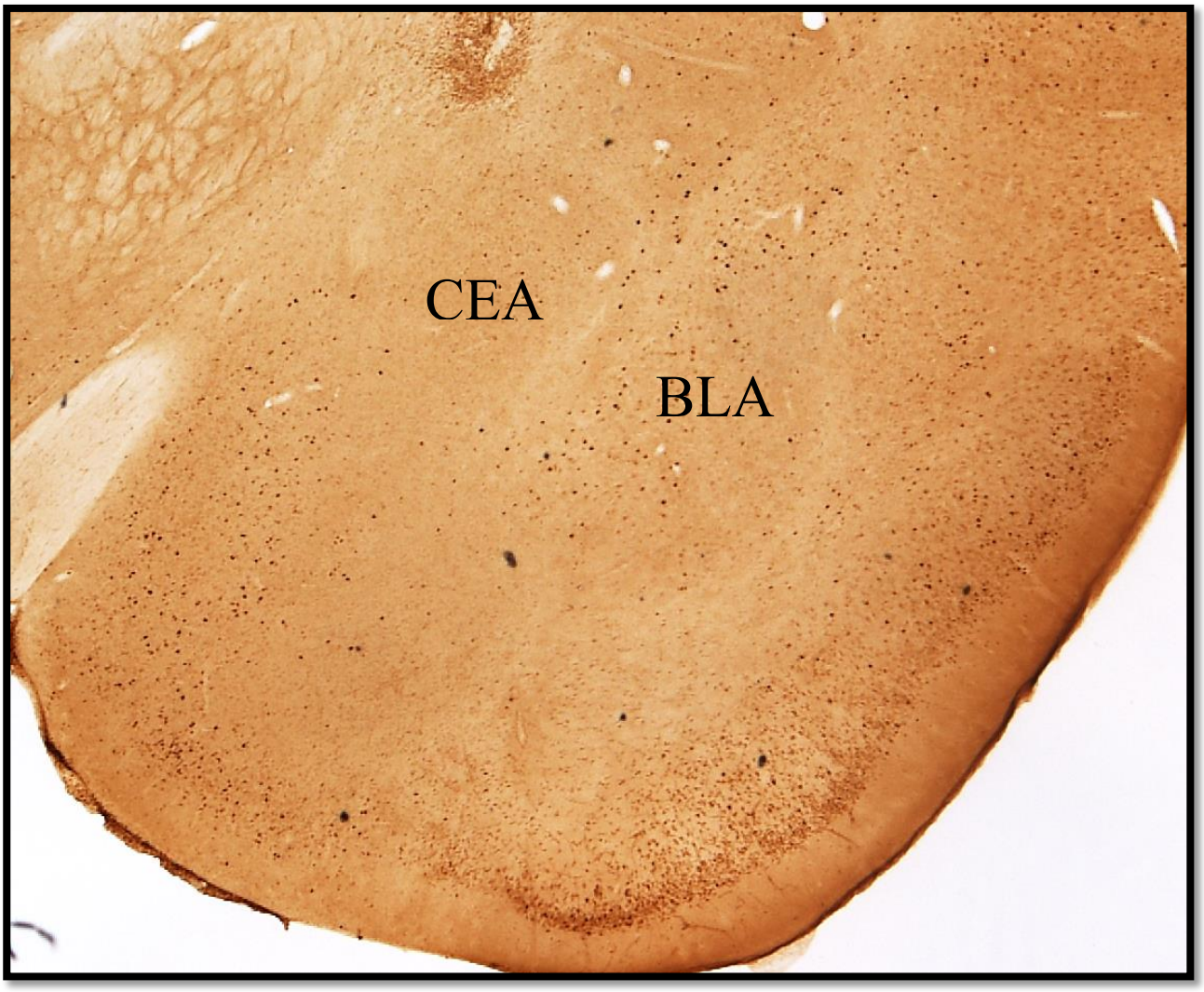


Figure 7